



**HAL**  
open science

**serovar Choleraesuis derivatives harbouring deletions in  
and regulatory genes are attenuated in pigs, and survive  
and multiply in porcine intestinal macrophages and  
fibroblasts, respectively**

Gustavo Domínguez-Bernal, Alberto Tierrez, Almira Bartolomé, Susana  
Martínez-Pulgarín, Francisco J. Salguero, José Antonio Orden, Ricardo de La  
Fuente

► **To cite this version:**

Gustavo Domínguez-Bernal, Alberto Tierrez, Almira Bartolomé, Susana Martínez-Pulgarín, Francisco J. Salguero, et al.. serovar Choleraesuis derivatives harbouring deletions in and regulatory genes are attenuated in pigs, and survive and multiply in porcine intestinal macrophages and fibroblasts, respectively. *Veterinary Microbiology*, 2008, 130 (3-4), pp.298. 10.1016/j.vetmic.2008.01.008 . hal-00532388

**HAL Id: hal-00532388**

**<https://hal.science/hal-00532388>**

Submitted on 4 Nov 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

Title: *Salmonella enterica* serovar Choleraesuis derivatives harbouring deletions in *rpoS* and *phoP* regulatory genes are attenuated in pigs, and survive and multiply in porcine intestinal macrophages and fibroblasts, respectively

Authors: Gustavo Domínguez-Bernal, Alberto Tierrez, Almira Bartolomé, Susana Martínez-Pulgarín, Francisco J. Salguero, José Antonio Orden, Ricardo de la Fuente

PII: S0378-1135(08)00026-6  
DOI: doi:10.1016/j.vetmic.2008.01.008  
Reference: VETMIC 3943

To appear in: *VETMIC*

Received date: 13-11-2007  
Revised date: 16-1-2008  
Accepted date: 18-1-2008

Please cite this article as: Domínguez-Bernal, G., Tierrez, A., Bartolomé, A., Martínez-Pulgarín, S., Salguero, F.J., Orden, J.A., de la Fuente, R., *Salmonella enterica* serovar Choleraesuis derivatives harbouring deletions in *rpoS* and *phoP* regulatory genes are attenuated in pigs, and survive and multiply in porcine intestinal macrophages and fibroblasts, respectively, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.01.008

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



1

2

***Salmonella enterica* serovar Choleraesuis derivatives**

3

**harbouring deletions in *rpoS* and *phoP* regulatory genes are**

4

**attenuated in pigs, and survive and multiply in porcine**

5

**intestinal macrophages and fibroblasts, respectively**

6

7

8

**Gustavo Domínguez-Bernal <sup>\*,1</sup>, Alberto Tierrez <sup>1,2</sup>, Almira Bartolomé <sup>1</sup>, Susana**

9

**Martínez-Pulgarín <sup>1</sup>, Francisco J. Salguero <sup>3</sup>, José Antonio Orden <sup>1</sup> and Ricardo de la**

10

**Fuente <sup>1</sup>**

11

12

13

<sup>1</sup> Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de

14

Madrid, 28040, Madrid, Spain.

15

<sup>2</sup> Present address: Centro Nacional de Investigaciones Cardiovasculares (Instituto Carlos III), Melchor Fernández Almagro 3, 28029, Madrid, Spain.

16

17

<sup>3</sup> Present address: Veterinary Laboratories Agency – Weybridge. Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom.

18

19

20

21

\*Corresponding author: G. Domínguez-Bernal. Departamento de Sanidad Animal, Facultad

22

de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain. Tel.: +34-

23

913943712. Fax: +34-913943908. E-mail: [gdbernal@vet.ucm.es](mailto:gdbernal@vet.ucm.es)

1 **Abstract**

2

3 Live attenuated *Salmonella enterica* strains have been extensively studied as  
4 potential vectors for the oral delivery of heterologous antigens. Due to its ability to target  
5 immune cells, its specific mechanism for crossing the intestinal barrier, and its swine-  
6 restricted tropism, *Salmonella enterica* subspecies *enterica* serovar Choleraesuis  
7 (*S. Choleraesuis*) has attracted a great deal of interest for the production of bacterial-based  
8 oral carriers specifically adapted to swine. In this study, two mutants of *S. Choleraesuis*  
9 were constructed and their attenuation and intracellular fate analysed with the purpose of  
10 engineering new attenuated live strains with improved properties as oral vaccine carriers.  
11 Those strains harboured a specific deletion either within the *phoP* or *rpoS* genes, which  
12 encode virulence-related regulators in *S. Typhimurium*. In comparison to the wild-type  
13 parental *S. Choleraesuis*, the mutant strains, especially  $\Delta phoP$ , were extremely low in  
14 virulence in the murine model and in the natural host, the pig. Moreover, when compared  
15 with a commercial live vaccine strain, SC-54, the two mutants showed a higher level of  
16 attenuation in mice and  $\Delta phoP$  also in pigs. In addition,  $\Delta rpoS$  and  $\Delta phoP$  presented a  
17 proliferation and survival phenotype within swine intestinal primary fibroblast and  
18 macrophage cell cultures, respectively. Collectively, the present results indicate that the  
19  $\Delta rpoS$  and  $\Delta phoP$  strains of *S. Choleraesuis* gather adequate features to be potential  
20 candidates for vaccine vectors for the specific delivery of heterologous antigens adapted to  
21 pigs.

22

23 **Key words:** *Salmonella* Choleraesuis; survival; proliferation; attenuation; pigs; vaccine vector.

24 **Abbreviated article title:** *rpoS* and *phoP* mutants of *S. Choleraesuis* as candidates for  
25 **antigen vehicles**

## 1 **1. Introduction**

2

3 *Salmonella enterica* subspecies *enterica* serovar Choleraesuis (*S. Choleraesuis*) is a  
4 host-adapted pathogen that causes systemic disease in pigs (Chiu et al., 2004).  
5 *S. Choleraesuis* is a facultative intracellular Gram-negative pathogen, that shows the  
6 invasion of eukaryotic cells, intracellular survival and replication in infected host cells  
7 which are hallmarks of *Salmonella* pathogenesis. After oral ingestion, *Salmonella* initiates  
8 infection in the ileal mucosa by crossing epithelial or M cells to reach and enter resident  
9 macrophages and dendritic cells (Jones et al., 1994). Whether the outcome of these  
10 interactions is a localized intestinal infection or systemic spread of the pathogen is  
11 determined by genetic factors of both the host species and the *Salmonella* serovar (Santos  
12 and Baumler, 2004). Systemic serovars are transported to the mesenteric lymph nodes by  
13 mononuclear phagocytes, followed by further dissemination to internal organs causing, in  
14 the case of *S. Choleraesuis*, septicemia in pigs that shows some similarity to that caused  
15 by *S. Typhimurium* in mice and *S. Typhi* in humans (Reed et al., 1986).

16 Attenuated strains of *S. Choleraesuis* have attracted a great deal of interest as potential  
17 live oral vaccine carriers for antigen delivery systems adapted to swine (Ku et al., 2005;  
18 Mastroeni et al., 2001; Mollenkopf et al., 2001). It is crucial that these attenuated  
19 *Salmonella* strains cross the epithelial layer and reach the appropriate local lymphoid cells  
20 in order to trigger the necessary signals that lead to the desired immune response. The  
21 intestinal lamina propria is filled with distinct subpopulations of fibroblasts (Adegboyega  
22 et al., 2002) that have been shown to be pivotal cells in the modulation of immune  
23 responses (Buckley et al., 2001). Hence, the ability to survive and proliferate in these cells  
24 would increase the suitability of vector candidates and may be a prerequisite for  
25 engineering new candidates. The available vaccines from the United States failed to pass

1 safety tests in Taiwan and the identification of improved attenuated strains is required.  
2 Thus, two new attenuated vaccine strains of *S. Choleraesuis* have been developed for use  
3 in their natural host (Chiu et al., 2004; Ku et al., 2005). Our novel strategy for the  
4 development of an oral *S. Choleraesuis* vector was to generate a mutation that combines a  
5 severe attenuation in virulence with the potential to survive in macrophages and proliferate  
6 in target host cells in order to enhance its immunogenicity. The rationale for designing  
7 *phoP* and *rpoS* mutations of *S. Choleraesuis* was derived from information obtained from  
8 equivalent mutations in *S. Typhimurium*, where interesting properties such as attenuation  
9 in the murine typhoid model and a proliferation phenotype in fibroblast cell cultures have  
10 been reported (Cano et al., 2001; Tierrez and Garcia-del Portillo, 2005).

11 With the aim of developing new carrier vectors highly adapted to the pig, in this  
12 study we developed two highly attenuated strains of *S. Choleraesuis*,  $\Delta phoP$  and  $\Delta rpoS$ .  
13 They were evaluated for their attenuation in mice and in the natural host by comparison  
14 with their parental wild-type (WT) strain and a commercially available vaccine strain, SC-  
15 54 (Roof and Doitchinoff, 1995). The interaction of *rpoS* and *phoP* mutants were further  
16 characterised in live primary swine intestinal fibroblasts and macrophages.

17

## 18 **2. Materials and methods**

19

### 20 *2.1. Bacterial strains and growth conditions*

21

22 The bacterial strains used in this study are listed in Table 1. *S. Choleraesuis* CECT  
23 915 (ATCC 13312) was used as the wild-type strain. Bacteria were routinely cultured in  
24 Luria-Bertani (LB) broth and on LB agar plates. If required for the selection of  
25 recombinants or to maintain plasmids, kanamycin (Km) 50 µg/ml and ampicillin (Amp)

1 100 µg/ml were added to the agar. The reference vaccine strain was a commercially  
2 available live avirulent culture (Enterisol<sup>®</sup> SC-54) and was obtained from Boehringer  
3 Ingelheim.

4

#### 5 2.2 Construction of *S. Choleraesuis* mutant strains

6

7 Specific *rpoS* and *phoP* gene disruptions were carried out as previously described  
8 (Datsenko and Wanner) with minor modifications: *S. Choleraesuis* transformants carrying  
9 a Red helper plasmid (pKD46) were grown in 50 ml LB cultures containing Amp 50  
10 µg/ml and L-arabinose (1 mM) at 30°C to an OD<sub>600</sub> of ≈ 0.6. They were then made  
11 electrocompetent by concentrating them 1000-fold following four washes with ice-cold  
12 double-distilled water (DDW). All PCR procedures were carried out according to standard  
13 protocols (Ausubel et al., 1992). PCR products were first precipitated with Pellet Paint<sup>™</sup>  
14 Co-Precipitant (Novagen), then purified from the agarose gel using the SpinPrep<sup>™</sup> Gel  
15 DNA Kit (Novagen), digested with *DpnI* to remove remaining methylated DNA, re-  
16 purified with the SpinPrep<sup>™</sup> PCR Clean-up Kit (Novagen) and eluted with DDW  
17 (Datsenko and Wanner, 2000). Electroporation was performed using a MicroPulser (Bio-  
18 Rad) with 0.2 cm chambers according to conditions specified by the manufacturer for  
19 *E. coli*, using 50 µl of bacteria and 300–500 ng of PCR product. 1 ml of SOC was added  
20 to the shocked bacteria, which were then incubated at 37°C for 1 h. Following  
21 concentration by centrifugation, bacteria were spread onto LB-Km-agar plates to select  
22 Km<sup>R</sup> transformants. Finally, when required, the FRT-flanked antibiotic resistance cassette  
23 was eliminated after transformation with pCP20, as previously described (Datsenko and  
24 Wanner, 2000). The PCR products that contained the Km-resistance gene flanked by  
25 sequences upstream and downstream of the disrupted genes were obtained using the

1 following primers: *rpoS*-1D and *rpoS*-1R were used to disrupt *rpoS*; *phoP*-1D and *phoP*-  
2 1R were employed to disrupt *phoP* (Table 2). All mutations were verified by sequencing  
3 following PCR amplification using locus specific primers: *rpoSN*-1D and *rpoSC*-1R for  
4 the *rpoS* junction locus; and *phoPN*-1D /*phoPC*-1R for the *phoP* region.

### 6 2.3. Isolation of primary swine intestinal macrophage and fibroblast cells

7  
8 Swine intestinal lamina propria macrophages (SIM) were isolated using enzymatic  
9 techniques as previously described (Sato et al., 2004). Briefly, mucosal strips were  
10 incubated at 37°C for three periods of 30 min in Hanks' balanced salt solution (HBSS)  
11 without calcium and magnesium and containing 2% fetal bovine serum (FBS), 1 mM  
12 dithiothreitol and 1.5 mM EDTA. During this treatment, mucus and epithelium were  
13 removed from the mucosa. The lamina propria containing macrophages were then  
14 chopped up and transferred into RPMI supplemented with 2% FBS and 0.4 Wünsch  
15 Units/ml collagenase (Blendzyme I, Roche). After 30 min incubation at 37°C the sample  
16 was passed through a 100- $\mu$ m filter mesh (cell strainer; Falcon, Becton Dickinson  
17 Labware, Franklin Lakes, NJ) to remove aggregates and then incubated for a further 30  
18 min in fresh collagenase solution. Pellets from the first and second incubations with  
19 collagenase were pooled, passed through a 100- $\mu$ m filter mesh, washed twice with RPMI  
20 containing 2% FBS, and the cells were then centrifuged over a Ficoll-Hypaque density  
21 gradient. Cells in the top Ficoll-Hypaque layer interface were recovered, washed twice and  
22 resuspended in 10 ml RPMI containing 10% FBS, 0.625 mM HEPES, 2 mM L-glutamine,  
23 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 0.25 $\mu$ g/ml fungizone. To obtain the  
24 macrophages, the cells were placed in tissue culture dishes at 37°C in 5% CO<sub>2</sub> for 1 hour  
25 and non-adherent cells were removed. The purity of the resulting SIM was confirmed by



1 flow-cytometry and immunofluorescence techniques utilizing the monocyte–macrophage  
2 marker CD14 (mouse anti-porcine CD14 AbD serotec).

3 Swine intestinal fibroblasts (SIF) were obtained as explants of surgically resected  
4 intestinal mucosa following a previously reported procedure (Strong et al., 1998). Briefly,  
5 mucus and epithelial cells were removed from mucosal strips as described above. Tissue  
6 was then cut into 0.2 cm x 0.2 cm pieces using a scalpel and 10 to 15 pieces per well were  
7 placed on coverslips in a 6-well tissue culture dish filled with 2 ml of Dulbecco’s minimal  
8 essential medium supplemented with 10% FBS, 0.625 mM HEPES, 2 mM L-glutamine,  
9 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone. The samples were  
10 incubated at 37°C in 5% CO<sub>2</sub>, replacing the medium twice a week. Once the fibroblasts  
11 had grown to subconfluence they were split and established as long-term cultures that  
12 were fed twice a week and split at confluence.

13

#### 14 2.4. Infection experiments

15

16 For infection of SIF and SIM cells the different *S. Choleraesuis* strains were grown to  
17 stationary phase (18 h) in LB broth at 37°C under static conditions, and 5 µl of each  
18 culture was added to eukaryotic cells cultured in medium free of antibiotic and antifungal  
19 agents in 24-well plastic plates to 50–60% confluency. The bacterial inocula were adjusted  
20 to a multiplicity of infection of 10:1 in the case of SIM and 50:1 in SIF  
21 (bacterial/eukaryotic cell ratio). Cells were infected for 10 min (SIM) or 20 min (SIF).  
22 After infection, the cells were washed three times with PBS, pH 7.4 (Invitrogen) with 10%  
23 FBS to remove noninternalized bacteria. Fresh growth medium containing 100 µg/ml of  
24 gentamicin was then added and maintained for up to 2 h postinfection (p.i.); the medium  
25 was then replaced with fresh medium containing 10 µg/ml of gentamicin for the remainder

1 of the experiment. For the enumeration of intracellular bacteria, eukaryotic cells were  
2 washed three times with PBS containing 10% FBS and lysed with 0.2% Triton X-100 in  
3 PBS for 5 min at 37°C; serial dilutions were plated onto LB agar plates. All samples were  
4 run at least in duplicate. The invasion rate was calculated as the percentage of the  
5 inoculum that became intracellular bacteria at 10 (SIM) or 20 (SIF) min post-infection.

### 6 7 2.5. Immunofluorescence

8  
9 For immunofluorescence analyses, the cells were grown in 24-well tissue culture  
10 plates on glass coverslips. After infection and incubation for the indicated time period, the  
11 cells were fixed with 3% paraformaldehyde in PBS for 10 min at room temperature and  
12 then washed three times with PBS and stored at 4°C until required. Prior to staining, the  
13 cells were blocked and permeabilized in a blocking solution consisting of 2% goat normal  
14 serum and 0.2% saponin (Sigma) in PBS for 10 min at room temperature. The coverslips  
15 were incubated with various antibodies diluted in the same blocking solution as detailed  
16 below, and were washed three times with PBS after each incubation step. The coverslips  
17 were mounted on Prolong Gold Antifade (Molecular Probes) and sealed with nail varnish.  
18 Samples were analyzed using a fluorescence microscope (ECLIPSE TE 200, Nikon). All  
19 antibody incubations were performed for 1 h at room temperature. Antisera and antibodies  
20 were used at the indicated dilutions: rabbit *Salmonella* O antiserum Poly B (BD) (1:500),  
21 and mouse anti-porcine CD14 (AbD serotec) (1:20). The following conjugates were used  
22 for detection at the indicated dilutions: goat anti-rabbit Alexa 488 conjugate (1:500); goat  
23 anti-mouse Alexa 488 (1:500); goat anti-mouse Alexa 594 (1:500). DAPI (4, 6-diamino-2-  
24 phenylindole; 1 µg/ml) was added for 5 min to stain the nuclei. For dead/alive studies,  
25 cells were incubated in the presence of propidium iodide (2 µg/ml) at room temperature

1 for 30 min prior to cell permeabilization and staining. Images were digitally collected and  
2 processed using Adobe Photoshop Software.

### 3 4 2.6. Competition assays

#### 5 6 2.6.1. *In vitro* competition assays

7  
8 To perform *in vitro* competition experiments, mutants (*phoP*::km, *rpoS*::km), WT  
9 and the commercially available SC-54 vaccine strain were grown separately in LB at 37°C  
10 overnight at 175 rpm. 1 ml of overnight mutant and WT or SC-54 strain cultures were  
11 mixed together at a ratio of 1:1 (1 OD<sub>600</sub> unit/ml each strain) and subcultured at 1:100 in  
12 50 ml of LB broth. Input ratios of mutant/WT or mutant/SC-54 were determined at this  
13 time. The cultures were grown in a shaker at 37°C for 24 h and then plated onto selective  
14 media to determine the output ratios of mutant to WT or SC-54. The competitive index  
15 (CI) was calculated for each mutant as the ratio between the mutant and WT or SC-54  
16 strain in the output (24 h) divided by their ratio in the input (initial mix) (Beuzon and  
17 Holden, 2001).

#### 18 19 2.6.2. *In vivo* competition assays in the murine model

20  
21 Eight-week-old female BALB/c mice (Charles Rivers Laboratories) were subjected  
22 to mixed infections. All mice were treated in accordance with the institutional guidelines  
23 for treatment of animals approved by the Ethical Committee for the Animal  
24 Experimentation, CEA, of the Complutense University of Madrid. Groups of three  
25 animals were inoculated via the intraperitoneal (i.p.) route with 10<sup>6</sup> and 10<sup>7</sup> colony  
26 forming units (CFU) of WT or SC-54 and mutant strains, respectively, in 0.2 ml of PBS.

1 Mice were sacrificed at 3 days post infection or when in a moribund condition. Spleens  
2 were aseptically recovered and ground by rubbing in 5 ml of cold PBS. 100 µl of the  
3 homogenate was serially diluted in PBS and plated on LB and LB with antibiotics for  
4 bacterial enumeration. The CI for each mutant was calculated as the ratio between the  
5 mutant and the WT or SC-54 strains in the output (bacteria recovered from the host after  
6 infection) divided by their ratio in the input (initial inoculum).

### 7 8 2.6.3. *In vivo* competition assays in the natural host

9  
10 Twelve 5- to 6-week-old crossbred (Landrace x Large White) piglets were obtained  
11 from a farm in Catalonia, Spain that was certified to be free of PRRS (porcine  
12 reproductive and respiratory syndrome), pseudorabies, and classical and African swine  
13 fever viruses. The animals were randomly divided into four groups of three, housed under  
14 pathogen-free conditions and allowed to acclimatize to the biosafety level 3 (BSL3)  
15 animal facility at the Centro de Investigación en Sanidad Animal, INIA, Madrid, for 1  
16 week before use in our experiments. All experiments with live animals were performed  
17 under the guidelines of the European Community (86/609) and were approved by the site  
18 ethical review committee. Rectal fecal swabs were taken prior to inoculation and were  
19 cultured for *Salmonella* by streaking onto Brilliant Green (BGA) and McConkey agar  
20 plates directly and after 24 h enrichment in tetrathionate broth. Isolates on McConkey agar  
21 that had colony morphology compatible with *Salmonella* spp. were streaked on BGA.  
22 Colonies on BGA with morphology compatible with *Salmonella* spp. were further  
23 screened by PCR. None of the fecal isolates was identified as *Salmonella*.

24 Feed was withdrawn from the pigs overnight prior to inoculation. Animals were  
25 lightly anaesthetized with ketamine HCl (Zoletyl 2 ml/pig) and restrained by hand, and the  
26 mouth was opened to allow gastric gavage. Ten to twenty min prior to inoculation the

1 stomach acidity was neutralized with 25 ml of 10% (wt/vol) sodium bicarbonate solution  
2 by gastric gavage with a 5 mm diameter Rusch naso-gastric tube (EMP), as previously  
3 described by (Kennedy et al., 1999). Pigs were inoculated by gastric gavage via the same  
4 tube with approximately  $10^8$  CFU (WT or SC-54) and  $10^9$  (any mutant) CFU (1:10 ratio)  
5 diluted in 25 ml of PBS. Bacterial inocula for inoculation of the pigs were grown in LB  
6 broth under static conditions to a cell density with an  $OD_{600}$  of approximately 0.5 (18 h).  
7 Inoculations were made based on cell density and were then measured by colony counts  
8 after serial dilution on LB/LB supplemented with kanamycin. All animals were euthanized  
9 3 days after inoculation and necropsies were performed as soon as possible after death.  
10 Samples (1 gram) of liver, spleen and ileocaecal mesenteric lymph nodes were collected,  
11 minced and homogenized in 5 ml PBS (pH 7.4). The bacterial load was determined by  
12 plating serial dilutions of the homogenate onto selective plating media. The CI was  
13 calculated as described above.

14

### 15 2.7. Statistical analysis

16

17 The mouse competitive index (CI) data were analyzed using the one sample Student's  
18 *t*-test. The null hypothesis was that the CI was not significantly different from 1. Data  
19 from the survival experiments were arithmetic log transformed and analysis was  
20 performed using one-way ANOVA and a posterior Tukey-Kramer multiple comparison  
21 test with a 95% confidence interval. In all cases differences were considered statistically  
22 significant when *p* was  $< 0.05$ . Analyses were performed using GraphPad InStat software  
23 (version 3.05).

24

## 25 3. Results

1

2 *3.1. Construction and characterization of rpoS or phoP mutants*

3

4 In this study we generated mutants of the *phoP* or *rpoS* regulatory genes in  
5 *S. Choleraesuis* as described in material and methods. A cardinal point was the stability of  
6 the engineered mutants and the lack of antibiotic resistance markers. This would lead to an  
7 optimized performance of the candidates and achieve the minimal requirement for  
8 biological safety. No obvious difference was observed in the in vitro growth curves of the  
9 parent and mutant strains, implying that the deletion of the *phoP* or *rpoS* genes had no  
10 significant influence on the growth of *S. Choleraesuis* (Fig. 1). In addition, no  
11 discrepancies were observed when utilization of a panel of 50 carbohydrates was analyzed  
12 by Api 50CH (Biomérieux®), demonstrating that any differences in the behaviour of the  
13 mutants could not be attributable to differences in growth capability or housekeeping gene  
14 activities.

15

16 *3.2. Virulence attenuation of mutant strains in the murine model*

17

18 The competitive index (CI) is a sensitive measure of the relative degree of virulence  
19 attenuation of a particular mutant in mixed infection with the WT strain. (Lichtensteiger  
20 and Vimr, 2003). Firstly, in vitro CI experiments were performed to verify that the strains  
21 had no defects in competence under normal laboratory conditions. Neither of the two  
22 candidate attenuated mutants tested showed disadvantages in growth, as the in vitro CI  
23 were not substantially different from those calculated in comparison with WT or SC-54  
24 (Table 3). With the aim of analyzing the effect on virulence of the  $\Delta rpoS$  and  $\Delta phoP$   
25 strains, mixed infections of the mutant versus the WT were carried out in BALB/c mice

1 after i.p. inoculation. Both  $\Delta rpoS$  and  $\Delta phoP$  were outcompeted by the WT strain (Table  
2 3), consistent with significant attenuation of the mutant strains compared with the WT.  
3 Furthermore,  $\Delta phoP$  was significantly (10000 times) more attenuated than  $\Delta rpoS$ . To  
4 provide a more relevant analysis, both mutants were also compared with a commercially  
5 available vaccine (SC-54) used as a standard attenuated strain. The results were  
6 significantly lower than unity, suggesting that  $\Delta rpoS$  and especially  $\Delta phoP$  were  
7 extremely attenuated in comparison to both WT and SC-54 in the murine model of  
8 infection.

9

### 10 3.3. Virulence attenuation in the natural host model (pig)

11

12 In order to assess if  $\Delta phoP$  and  $\Delta rpoS$  were also attenuated when infecting the natural  
13 host, competition assays were performed after oral inoculation of pigs. For this analysis,  
14 mixed infections with the WT and mutant strains were used to provide an in vivo measure  
15 of virulence attenuation, referred to as the CI. The CI data indicated that  $phoP$  and  $rpoS$   
16 deletion mutants were highly attenuated for enteric infections following oral inoculation of  
17 pigs. Moreover, and according with the results obtained in mice, the attenuation of  $\Delta phoP$   
18 was 1000 times higher than  $\Delta rpoS$  (Table 4). None of the pigs showed clinical signs, with  
19 the exception of a mild fever. In all pigs, the WT was more fit for colonization of the  
20 ileocaecal lymph nodes, although no bacteria were recovered from the spleen or liver.  
21 These results emphasize the in vivo importance of  $\Delta rpoS$  and especially  $\Delta phoP$  in  
22 decreasing the pathogenicity of *S. Choleraesuis* in its natural host, the pig.

23 Finally, comparison of the attenuation of the mutant strains relative to the  
24 commercial vaccine strain, SC-54 revealed that  $\Delta phoP$  was highly attenuated while  $\Delta rpoS$

1 showed equivalent attenuation to SC-54 (Table 4). These data demonstrate that deletion of  
2 *rpoS* or *phoP* caused a strong attenuation of virulence in the natural host, which was  
3 higher in the case of  $\Delta phoP$  where even more attenuation than the SC-54 strain was found.

4  
5 *3.4. Intracellular survival of  $\Delta phoP$ ,  $\Delta rpoS$ , SC-54 and WT strains of S. Choleraesuis*  
6 *within porcine intestinal macrophages*

7  
8 We examined the ability of WT, SC-54,  $\Delta phoP$  and  $\Delta rpoS$  mutants to survive inside  
9 porcine intestinal primary macrophage cells (SIM) after phagocytosis. For all the strains,  
10 there was a decrease in the number of viable bacteria during the first 2 hours (Fig. 2A).  
11 After the initial decrease in titre, the survival rate of the  $\Delta phoP$  and  $\Delta rpoS$  mutants was  
12 similar to that of the parent strain (Fig. 2A). However, a significant difference in survival  
13 from that of the parent was evident with SC-54, which was readily cleared 2 hours after  
14 phagocytosis. The intracellular proliferation rate was estimated as the ratio of the number  
15 of viable intracellular bacteria present at 24 h to the number present at 2 h p.i. (Cano et al.,  
16 2001). Neither the *phoP* nor the *rpoS* mutant exhibited a significant difference in viable  
17 bacteria compared with the WT (Fig 2A, Table 5). These observations indicate that both  
18 mutants could represent excellent delivery vectors for immune target cells.

19  
20 *3.5. Invasion and proliferation of  $\Delta phoP$ ,  $\Delta rpoS$ , SC-54 and WT S. Choleraesuis strains in*  
21 *swine intestinal fibroblasts*

22  
23 The facts that *Salmonella* infects fibroblast cell lines in vitro and that there is a  
24 large population of fibroblasts in the gut led us to perform the isolation and later infection  
25 of primary fibroblasts from the intestinal mucosa. The aim was to continue to characterize



1 the candidate vaccine vectors by utilizing primary cell cultures that might reproduce more  
2 accurately the conditions found in vivo. For this purpose, we isolated, cultured, and  
3 expanded primary intestinal fibroblasts from pigs (SIF), following protocols previously  
4 described, with modifications (Strong et al., 1998). The invasion rates were not  
5 distinguishable among the mutants and the WT and SC-54 strains (Table 5).

6 We further analyzed the survival of WT, SC-54,  $\Delta phoP$  and  $\Delta rpoS$  strains when  
7 infecting SIF. In accordance with the results obtained from the macrophage infection  
8 experiments, all the strains suffered a loss in titre during the first 2 h of infection, which  
9 was substantially greater in the case of SC-54 (Fig. 2B). Also interesting was the  
10 significantly larger number of live bacteria of  $\Delta phoP$  and  $\Delta rpoS$  compared with WT (Fig.  
11 2B;  $p < 0.05$ ). Moreover, in contrast to their behaviour inside macrophages, the four  
12 strains showed a significant increase in the number of viable bacteria inside fibroblasts at  
13 24 h. The above results correlated with the intracellular proliferation rate of WT and the  
14 three attenuated strains inside SIF (Ipro<sub>wt</sub> 41.2±11.6; Ipro <sub>$\Delta phoP$</sub>  30.5±6.9; Ipro <sub>$\Delta rpoS$</sub>   
15 35.6±13.9 and Ipro<sub>SC-54</sub> 47.5±21.4) (Table 5). However, when comparing the behaviour of  
16 each strain at 24 h we found that the number of WT,  $\Delta phoP$  and  $\Delta rpoS$  was significantly  
17 higher than the number of SC-54 (Fig. 2B;  $p < 0.001$ ). Indeed, this increase was, in the  
18 case of  $\Delta rpoS$ , significantly greater than in WT (Fig. 2B;  $p < 0.05$ ).

19 Based on the above results, in order to further determine if the increases in  
20 bacterial load over 24 h was due to uniform bacterial proliferation inside all the infected  
21 cells or to higher proliferation in only a small proportion of cells, fibroblasts infected with  
22 *S. Choleraesuis* were stained for immunofluorescence. SIFs grown on coverslips were  
23 infected with the different strains and stained for the presence of *Salmonella* (Fig. 3). We  
24 verify that the increase in bacterial numbers occurred intracellularly in live cells. Dead  
25 cells were clearly identified by positive staining of the nuclei with propidium iodide and

1 morphological features (round shaped and refringent cells; Fig. 3B). The number of  
2 intracellular bacteria at 2 and 24 h p.i. was determined by counting one hundred live  
3 infected cells. Figure 4 shows the percentage of live cells infected with different numbers  
4 of bacteria (1–5, 6–20 and > 20) resulting from three separate experiments. The majority  
5 of the cells infected with WT (95.1%) harboured fewer than 5 bacteria 2 h post-infection  
6 (p.i.), and only 4.9% showed between 6 and 20 intracellular bacteria. Although the number  
7 of cells infected with 6–20 or more than 20 bacteria increased at 24 h p.i. (24.2% and  
8 12.7%, respectively), a high percentage of infected cells (63.1%) still contained fewer than  
9 5 bacteria (Fig. 4). Curiously, most of the cells that contained many bacteria 24 h p.i. were  
10 dead (data not shown, see Fig. 3 for an example). Whether a massive proliferation of the  
11 WT induces cell death or non-viable cells are permissive for bacterial growth remains to  
12 be investigated. Most interestingly, the  $\Delta phoP$  and  $\Delta rpoS$  strains behaved differently from  
13 the WT. Thus, although the number of intracellular bacteria at early time points was  
14 similar to that observed with the parental strain (75.2% and 71.9% of the cells infected  
15 with fewer than 5 bacteria, respectively), the findings observed at 24 h p.i. were  
16 consistently different: 69.6% of  $\Delta phoP$  and 79.9% of  $\Delta rpoS$  live infected cells harboured  
17 more than 20 bacteria, which were contained in large vacuoles. These results are  
18 consistent with an enhanced proliferation of  $\Delta phoP$  and  $\Delta rpoS$  in live fibroblasts (Figs. 3A  
19 and 4). Due to the extremely low number of live cells infected with the SC-54 strain at 2  
20 and 24 h p.i. no statistically significant results could be obtained. Although there were  
21 some cells in which SC-54 showed intracellular proliferation at 24 h p.i., it seemed that  
22 most of the bacteria were cleared, according to the frequent observation of bacterial cell  
23 wall remains inside the cells (data not shown).

24

25 **Discussion**

1

2 Attenuated live *S. Choleraesuis* is a versatile organism for the generation of live  
3 recombinant vectors for mucosal immunization, and is specifically adapted to the pig as its  
4 natural host (Chiu et al., 2004). A major drawback in the use of live attenuated  
5 intracellular bacteria as oral delivery vehicles can be a lack of survival or proliferation  
6 within the target immune cells. In this study, we constructed two new attenuated strains  
7 with excellent potential as carriers specifically developed for porcine antigen delivery.

8 Careful selection of an appropriate carrier strain and its attenuated mutations may  
9 represent an important means to fine-tune the quality of the immune response. One of the  
10 most widely studied classes of rationally attenuated *Salmonellae* are auxotrophs (Daudel  
11 et al., 2007); however, these mutants have been shown to be poorly immunogenic in mice.  
12 Another well studied group of attenuated *Salmonellae* are mutants bearing defined  
13 deletions in genes involved in the regulation of *Salmonella* virulence. Among these,  
14 vaccine strains with mutations in the *phoP* and *phoQ* genes, which are involved in the  
15 two-component regulatory system for  $Mg^{2+}$  sensing (Hohmann et al., 1996b), and  
16 alternative sigma factor (RpoS) (Coynault et al., 1996) have been constructed and assessed  
17 as candidate vaccines against murine or human typhoid fever. The PhoP/Q controlled gene  
18 products play important roles in the pathogenesis of *Salmonella* infection mainly by  
19 promoting resistance to innate immune defence mechanisms. RpoS is also recognized as a  
20 global stationary-phase sigma factor that controls the expression of many genes and is  
21 required for the virulence of *S. Typhimurium* and *S. Typhi* (Coynault et al., 1996).  
22 Although the properties of virulence factors cannot necessarily be extrapolated across  
23 *Salmonella* serovars, *rpoS* and *phoP* deletion mutants of *S. Choleraesuis* were constructed  
24 with the aim of developing strains adapted to the pig and exploiting them as live vectors  
25 for mucosal immunization. Interestingly, in agreement with the results previously  
26 described for *S. Typhimurium*, we found that mutations in the *phoP/Q* ( $\Delta$ *phoP*) system

1 and *rpoS* ( $\Delta rpoS$ ) of *S. Choleraesuis* showed dramatic virulence attenuation in the murine  
2 model of infection. This significant attenuation was further corroborated in the porcine  
3 natural model of infection. It is interesting to notice that, as previously reported for  
4 *S. Typhimurium* in mice (Hohmann et al., 1996b; Coynault et al., 1996), higher  
5 attenuation in  $\Delta phoP$  was found in both models of infection. However, this different level  
6 of attenuation between  $\Delta phoP$  and  $\Delta rpoS$  in the mice and pig models remains to be  
7 investigated in term of possible, if any, significance in vector induced immunity.  
8 *S. Choleraesuis* SC-54 is an attenuated commercial vaccine strain that is used to prevent  
9 piglet paratyphoid; it is attenuated by a chemical method (Roof and Doitchinoff, 1995).  
10 Although the vaccine has good immunogenicity, it retains some residual virulence (Ku et  
11 al., 2005). Unexpectedly, comparison of  $\Delta phoP$  and  $\Delta rpoS$  with SC-54 in the murine  
12 model of infection by determination of the CI revealed stronger virulence attenuation in  
13 our two engineered strains. More interesting is that this was also observed, in the case of  
14  $\Delta phoP$ , when the strains were tested in the porcine model of infection. Thus, the properties  
15 of both attenuated mutants in mice, and  $\Delta phoP$  in pigs, seem to be markedly better than  
16 the commercially available strain in terms of in vivo attenuation.

17 As a facultative intracellular pathogen, *Salmonella* has evolved to reside and  
18 replicate in dendritic cells (Hopkins et al., 2000) and in macrophages in the Peyer's  
19 patches and other lymphoid tissues of the small intestine, where a local mucosal immune  
20 response is triggered. The rapid uptake of bacteria into the lamina propria of the gut is  
21 important for eliciting an effective immune response. The data obtained in this report  
22 show how *phoP* and *rpoS* mutants survive as well within primary intestinal macrophages  
23 as the WT does, in clear contrast to the behaviour exhibited by the commercial vaccine  
24 strain SC-54, which is unable to survive in this type of immune cell. These results were  
25 unexpected for  $\Delta phoP$ , considering that the absence of the PhoP-PhoQ regulatory system

1 has previously been linked to major intramacrophage survival defects (Groisman and  
2 Ochman, 1997). These discrepancies could be due to differences in the assays of infection  
3 used, such as the use of cell lines vs. primary macrophage cell cultures, the genetic  
4 background of the eukaryotic cells, bacterial opsonization prior to infection or the  
5 activation state of the macrophages employed. Controversial data have been published in  
6 the case of *rpoS* mutants, with some authors showing it as essential for *S. Typhimurium*  
7 responses that are linked to resistance to macrophage attack (Cano et al., 2001; Coynault  
8 et al., 1996), and others reporting that, although attenuated, *rpoS* mutants could multiply  
9 inside cultured macrophages (Khan et al., 1998; Nickerson and Curtiss, 1997). The  
10 survival of *S. Choleraesuis* within porcine alveolar macrophages seems not to be directly  
11 correlated with its virulence in pigs; this is also supported by our data on attenuation,  
12 together with observations of survival in primary SIM (Paulin et al., 2007; Watson et al.,  
13 2000).

14 The lamina propria also contains distinct subpopulations of fibroblasts (Adegboyega et  
15 al., 2002). Fibroblasts have been shown to be pivotal cells in the modulation of immune  
16 responses (Buckley et al., 2001). It has been shown that *Salmonellae* do not replicate or  
17 cause cytotoxicity in fibroblasts but simply remain in a persistent state of infection (Cano  
18 et al., 2001). Moreover, evidence has recently been reported of the presence of  
19 *S. Typhimurium* in fibroblasts *in vivo*, shown by images of *S. Typhimurium* targeting  
20 nonphagocytic (CD45-negative) cells in the lamina propria of mice (Boyle et al., 2007).  
21 We have therefore investigated the intracellular fate of *S. Choleraesuis* within primary  
22 SIF. The determination of the proliferation rate by recording the number of recovered  
23 bacteria at 2 and 24 h p.i. showed a net increase in all the strains tested at 24 h (Figure 2B  
24 and Table 5). Nevertheless, the analysis of the bacterial load per host cell revealed that  
25 only *phoP* and *rpoS* mutants actually displayed a proliferation phenotype inside live  
26 eukaryotic cells (Fig. 4). The discrepancies between the two methods employed for

1 measuring bacterial proliferation inside fibroblasts could be explained by the fact that  
2 proliferation of the WT occurred mostly in dead cells, although numerous bacteria were  
3 also recorded in dead host cells in the case of  $\Delta phoP$  and  $\Delta rpoS$ . These unexpected  
4 findings could be explained by a lethal effect of the bacteria inside fibroblasts. However,  
5 this explanation seems less plausible, because many bacteria were also observed in live  
6 cells in the case of both mutants. Hence, overgrowth in previously dead or moribund cells  
7 that lack defences seems to be the most probable scenario. Therefore, only live eukaryotic  
8 cells were taken into account in our analysis. Under these conditions a proliferating  
9 phenotype was found for  $\Delta phoP$  and  $\Delta rpoS$  in primary fibroblast cell cultures obtained  
10 from the swine lamina propria, in clear contrast to the non-proliferating state shown by the  
11 WT and SC-54. It is relevant to notice that, unlike in *S. Typhimurium* where the  
12 proliferation values of *rpoS* were lower than those of the *phoP* mutant (Cano et al., 2001),  
13 in *S. Choleraesuis* both mutants exhibited similar behaviour. Whether this is related to the  
14 specific properties of the serovar, the non-immortalized cell lines employed, or the  
15 accuracy of the proliferation assay, in which only bacteria in live cells were recorded,  
16 remains to be elucidated.

17 In vitro, *Salmonella* replicates to high numbers within epithelial cells and macrophages,  
18 yet, in vivo, infected cells usually contain only one or two bacteria (Brown et al., 2006;  
19 Sheppard et al., 2003). Massive proliferation within fibroblast cells could result in a  
20 suicide strategy. Thus, lysis of infected fibroblasts that contain many *Salmonella* cells may  
21 lead to bacterial release into the extracellular spaces, from which the bacteria could be  
22 subsequently taken up by neighbouring dendritic cells. In this case, dendritic cells might  
23 act as bystander antigen-presenting cells for T-cells, thus increasing the immune response  
24 (Sundquist et al., 2004). These features, together with survival in intestinal macrophages,  
25 strongly encourage further investigation of  $\Delta phoP$  and  $\Delta rpoS$  as vaccine vector candidates.

1           The main conclusion to emerge from this work is that two novel attenuated strains  
2 of *S. Choleraesuis* could be considered as new candidates for promising live vaccine  
3 vectors, for which a combination of intracellular proliferation in porcine fibroblasts,  
4 maintenance of viability in porcine macrophages and attenuation in the porcine model of  
5 infection are important prerequisites. Furthermore, the  $\Delta phoP$  and  $\Delta rpoS$  strains, being  
6 highly adapted to swine as their natural host, might be used as excellent carriers of foreign  
7 antigens. In summary, use of these strains could open new avenues for the engineering of  
8 multivalent live vaccines for pigs.

## 10 **Acknowledgements**

12           This research was supported by a grant from the Spanish Ministry of Education and  
13 Science (MEC) (AGL2004-00087) and the Universidad Complutense-Comunidad de  
14 Madrid (920338). G. Domínguez-Bernal is an investigator of the "Ramón y Cajal" program  
15 from the MEC and A. Bartolomé was supported by a predoctoral fellowship from the  
16 MEC. We thank Dr. Cinta Prieto for the kind gifts of porcine samples and R. Sánchez and  
17 E. Neves for their technical support. We also thank Dr M.T. Ripio for her critical  
18 comments.

## 20 **Appendix**

22           The University Complutense of Madrid (Spain) has filed a patent on the  $\Delta rpoS$  strain as a  
23 carrier delivery vector.

## References

- Adegboyega, P.A., Mifflin, R.C., DiMari, J.F., Saada, J.I., Powell, D.W., 2002, Immunohistochemical study of myofibroblasts in normal colonic mucosa, hyperplastic polyps, and adenomatous colorectal polyps. *Arch Pathol Lab Med* 126, 829-836.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., 1992, *Current protocols in molecular biology*. Green Publishing Associates and Wiley-Interscience, New York, N.Y.
- Beuzon, C.R., Holden, D.W., 2001, Use of mixed infections with *Salmonella* strains to study virulence genes and their interactions in vivo. *Microbes Infect* 3, 1345-1352.
- Boyle, E.C., Bishop, J.L., Grassl, G.A., Finlay, B.B., 2007, *Salmonella*: from pathogenesis to therapeutics. *J Bacteriol* 189, 1489-1495.
- Brown, S.P., Cornell, S.J., Sheppard, M., Grant, A.J., Maskell, D.J., Grenfell, B.T., Mastroeni, P., 2006, Intracellular demography and the dynamics of *Salmonella enterica* infections. *PLoS Biol* 4, e349.
- Buckley, C.D., Pilling, D., Lord, J.M., Akbar, A.N., Scheel-Toellner, D., Salmon, M., 2001, Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol* 22, 199-204.
- Cano, D.A., Martinez-Moya, M., Pucciarelli, M.G., Groisman, E.A., Casadesus, J., Garcia-Del Portillo, F., 2001, *Salmonella enterica* serovar Typhimurium response involved in attenuation of pathogen intracellular proliferation. *Infect Immun* 69, 6463-6474.
- Chiu, C.H., Su, L.H., Chu, C., 2004, *Salmonella enterica* serotype Choleraesuis: epidemiology, pathogenesis, clinical disease, and treatment. *Clin Microbiol Rev* 17, 311-322.
- Coynault, C., Robbe-Saule, V., Norel, F., 1996, Virulence and vaccine potential of *Salmonella typhimurium* mutants deficient in the expression of the RpoS ( $\sigma$ S) regulon. *Mol Microbiol* 22, 149-160.
- Datsenko, K.A., Wanner, B.L., 2000, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97, 6640-6645.



- Daudel, D., Weidinger, G., Spreng, S., 2007, Use of attenuated bacteria as delivery vectors for DNA vaccines. *Expert Rev Vaccines* 6, 97-110.
- Groisman, E.A., Ochman, H., 1997, How *Salmonella* became a pathogen. *Trends Microbiol* 5, 343-349.
- Hohmann, E.L., Oletta, C.A., Miller, S.I., 1996b, Evaluation of a *phoP/phoQ*-deleted, *aroA*-deleted live oral *Salmonella typhi* vaccine strain in human volunteers. *Vaccine* 14, 19-24.
- Hopkins, S.A., Niedergang, F., Corthesy-Theulaz, I.E., Kraehenbuhl, J.P., 2000, A recombinant *Salmonella typhimurium* vaccine strain is taken up and survives within murine Peyer's patch dendritic cells. *Cell Microbiol* 2, 59-68.
- Jones, B.D., Ghori, N., Falkow, S., 1994, *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J Exp Med* 180, 15-23.
- Kennedy, M.J., Yancey, R.J., Jr., Sanchez, M.S., Rzepkowski, R.A., Kelly, S.M., Curtiss, R., 3rd, 1999, Attenuation and immunogenicity of Deltacya Deltacr<sub>p</sub> derivatives of *Salmonella choleraesuis* in pigs. *Infect Immun* 67, 4628-4636.
- Khan, A.Q., Zhao, L., Hirose, K., Miyake, M., Li, T., Hashimoto, Y., Kawamura, Y., Ezaki, T., 1998, *Salmonella typhi* *rpoS* mutant is less cytotoxic than the parent strain but survives inside resting THP-1 macrophages. *FEMS Microbiol Lett* 161, 201-208.
- Ku, Y.W., McDonough, S.P., Palaniappan, R.U., Chang, C.F., Chang, Y.F., 2005, Novel attenuated *Salmonella enterica* serovar *Choleraesuis* strains as live vaccine candidates generated by signature-tagged mutagenesis. *Infect Immun* 73, 8194-8203.
- Lichtensteiger, C.A., Vimr, E.R., 2003, Systemic and enteric colonization of pigs by a *hlaA* signature-tagged mutant of *Salmonella choleraesuis*. *Microb Pathog* 34, 149-154.
- Mastroeni, P., Chabalgoity, J.A., Dunstan, S.J., Maskell, D.J., Dougan, G., 2001, *Salmonella*: immune responses and vaccines. *Vet J* 161, 132-164.
- Mollenkopf, H., Dietrich, G., Kaufmann, S.H., 2001, Intracellular bacteria as targets and carriers for vaccination. *Biol Chem* 382, 521-532.
- Nickerson, C.A., Curtiss, R., 3rd, 1997, Role of sigma factor *RpoS* in initial stages of *Salmonella typhimurium* infection. *Infect Immun* 65, 1814-1823.

- Paulin, S.M., Jagannathan, A., Campbell, J., Wallis, T.S., Stevens, M.P., 2007, Net replication of *Salmonella enterica* serovars Typhimurium and Choleraesuis in porcine intestinal mucosa and nodes is associated with their differential virulence. *Infect Immun*.
- Reed, W.M., Olander, H.J., Thacker, H.L., 1986, Studies on the pathogenesis of *Salmonella typhimurium* and *Salmonella choleraesuis* var *kunzendorf* infection in weanling pigs. *Am J Vet Res* 47, 75-83.
- Roof, M.B., Doitchinoff, D.D., 1995, Safety, efficacy, and duration of immunity induced in swine by use of an avirulent live *Salmonella choleraesuis*-containing vaccine. *Am J Vet Res* 56, 39-44.
- Santos, R.L., Baumber, A.J., 2004, Cell tropism of *Salmonella enterica*. *Int J Med Microbiol* 294, 225-233.
- Sato, T., Kanai, T., Watanabe, M., Sakuraba, A., Okamoto, S., Nakai, T., Okazawa, A., Inoue, N., Totsuka, T., Yamazaki, M., Kroczeck, R.A., Fukushima, T., Ishii, H., Hibi, T., 2004, Hyperexpression of inducible costimulator and its contribution on lamina propria T cells in inflammatory bowel disease. *Gastroenterology* 126, 829-839.
- Sheppard, M., Webb, C., Heath, F., Mallows, V., Emilianus, R., Maskell, D., Mastroeni, P., 2003, Dynamics of bacterial growth and distribution within the liver during *Salmonella* infection. *Cell Microbiol* 5, 593-600.
- Strong, S.A., Pizarro, T.T., Klein, J.S., Cominelli, F., Fiocchi, C., 1998, Proinflammatory cytokines differentially modulate their own expression in human intestinal mucosal mesenchymal cells. *Gastroenterology* 114, 1244-1256.
- Sundquist, M., Rydstrom, A., Wick, M.J., 2004, Immunity to *Salmonella* from a dendritic point of view. *Cell Microbiol* 6, 1-11.
- Tierrez, A., Garcia-del Portillo, F., 2005, New concepts in *Salmonella* virulence: the importance of reducing the intracellular growth rate in the host. *Cell Microbiol* 7, 901-909.
- Watson, P.R., Paulin, S.M., Jones, P.W., Wallis, T.S., 2000, Interaction of *Salmonella* serotypes with porcine macrophages in vitro does not correlate with virulence. *Microbiology* 146, 1639-1649.

**Table 1. Bacterial strains and plasmids used in this study**

Strain or plasmid	Description and relevant characteristics	Source (Reference)
<i>Salmonella enterica</i> serovar Choleraesuis		
CECT <sup>a</sup> 915	Wild-type	Spanish Culture Type Collection
SCL <sup>b</sup> 00022	<i>rpoS</i> ::km from CECT 915, Km <sup>R</sup>	This work
SCL 00023	<i>phoP</i> ::km from CECT 915, Km <sup>R</sup>	This work
SCL 00031	$\Delta$ <i>rpoS</i> from CECT 915	This work
SCL 00032	$\Delta$ <i>phoP</i> from CECT 915	This work
SCL 00021	Wild-type / pKD46, Amp <sup>R</sup>	This work
SC-54	Commercial Live Vaccine Strain	(Boehringer Ingelheim) (Roof and Doitchinoff, 1995)
Plasmids		
pKD46	Expressing Red, ts; Amp <sup>R</sup>	(Datsenko and Wanner, 2000)
pCP20	Expressing FLP, ts; Amp <sup>R</sup>	(Datsenko and Wanner, 2000)

<sup>a</sup> CECT, Spanish collection of Type Cultures, Universidad de Valencia, Burjassot (Valencia), Spain.

<sup>b</sup> SCL, *Salmonella* Choleraesuis collection from the INBAVET group.

**Table 2.** Primers used in this study

Primer designation	Sequence (5'→3')	Orientation	Template plasmid/Gene target
<b>rpoS-1D*</b>	TAGTTCGGTCAAGGGATCACGGGTAGGAGC CACCTTGTGTAGGCTGGAGCTGCTTC	Forward	pKD4
<b>rpoS-1R*</b>	GTCGACAGACTGGCCTTTTTTTGACAAGGGT ACTTACATATGAATATCCTCCTTAG	Reverse	pKD4
<b>phoP-1D*</b>	CTAGACTGTTCTTATTGTTAACACAAGGGAG AAGAGGTGTAGGCTGGAGCTGCTTC	Forward	pKD4
<b>phoP-1R*</b>	GCGACAGCGGCAGAAAATGGCGAGCAAATT TATTCATTACATATGAATATCCTCCTTAG	Reverse	pKD4
<b>rpoSN-1D</b>	GCACCGGCACCAGCTCTACAC	Forward	<i>nlpD</i>
<b>rpoSC-1R</b>	CCAGCCGGAACACTATCCAC	Reverse	Intergenic region downstream <i>rpoS</i>
<b>phoPN-1D</b>	AGAGGGTGACTATTGTCTGG	Forward	Intergenic region upstream <i>phoP</i>
<b>phoPC-1R</b>	CAGGTTGCTTTCGCCGCGCAG	Reverse	<i>phoQ</i>

The specific plasmid sequences are depicted in bold.

\*Primers were designed according to (Datsenko and Wanner, 2000). 3' regions marked in bold anneal to the pKD4 plasmid template. Extensions of their 5' regions are homologous to the target sequences for recombination.

**Table 3.** Competitive indexes of attenuated strains in BALB/c mice

Strains compared	Challenge route	In vitro <sup>1</sup>	CI <sup>3</sup> (SE)		Attenuation
			In vivo <sup>2</sup>	<i>p</i> <sup>4</sup>	
<i>phoP</i> ::km vs. WT	i.p.	1.25 (± 0.42)	0.00000015 (± 0.00000019)	< 0.0002	yes
<i>rpoS</i> ::km vs. WT	i.p.	0.96 (± 0.22)	0.0028 (± 0.0016)	< 0.0002	yes
<i>phoP</i> ::km vs. SC-54	i.p.	1.24 (± 0.68)	0.0000094 (± 0.0000073)	< 0.0002	yes
<i>rpoS</i> ::km vs. SC-54	i.p.	1.09 (± 0.77)	0.018 (± 0.010)	< 0.0002	yes

<sup>1</sup> 1:1 Mixtures of WT or SC-54 with each mutant were adjusted to an initial OD<sub>600</sub> = 0.03. The mixed cultures were maintained at 37°C with shaking for 24 h.

<sup>2</sup> Only animals in a moribund state were considered.

<sup>3</sup> CI it is the ratio of mutant to wild-type *Salmonella*, corrected for the inoculum ratio, recovered from the spleen upon i.p. challenge of BALB/c mice. Data show mean ± standard error of the mean (SE) obtained from a minimum of three mice from three independent experiments. Competitive index values lower than 0.4 are considered to represent defects in competence and virulence in vitro (Lichtensteiger and Vimr, 2003)

<sup>4</sup> *p*-value for one sample *t*-test for CI equal to 1.

**Table 4.** Competitive indexes of attenuated strains in pigs

Strains compared	Challenge route	CI (SE) <sup>1</sup>	P <sup>2</sup>	Attenuation <sup>3</sup>
<i>phoP</i> ::km vs. WT	oral	0.000013 <sup>4</sup>	N.D.	yes
<i>rpoS</i> ::km vs. WT	oral	0.022 (± 0.006)	< 0.0001	yes
<i>phoP</i> ::km vs. SC-54	oral	0.00011 (± 0.00015)	< 0.0001	yes
<i>rpoS</i> ::km vs. SC-54	oral	1.31 (± 1.5)	< 0.7518	no

<sup>1</sup> Competitive index (CI), it is the ratio of mutant to wild-type *Salmonella* recovered from ileocaecal mesenteric lymph node homogenates of inoculated pigs (three pigs in each group) corrected for the inoculum ratio. Data show mean ± standard error of the mean (SE)

<sup>2</sup> *p*-value for one sample *t*-test for CI equal to 1.

<sup>3</sup> Mutants are attenuated for colonization of tissues if CI < 0.4.

<sup>4</sup> Data obtained from one animal due to the lack of bacteria in the rest of animals from the group.

**Table 5.** Invasion and intracellular growth rates in SIM and SIF

	SIM		SIF	
	% internalization <sup>1</sup>	I <sub>pro</sub> rate <sup>2</sup>	% invasion <sup>1</sup>	I <sub>pro</sub> rate <sup>2</sup>
<b>WT</b>	0.65 ± 0.15	2.1 ± 1.3	1.49 ± 0.62	41.2 ± 11.6
<b><i>ΔphoP</i></b>	0.86 ± 0.35	4.8 ± 2.9	1.36 ± 0.31	30.5 ± 6.9
<b><i>ΔrpoS</i></b>	0.90 ± 0.63	3.8 ± 1.5	1.72 ± 0.29	35.6 ± 13.9
<b>SC-54</b>	0.84 ± 0.42	0.2 ± 0.4	1.14 ± 0.58	47.5 ± 21.4

<sup>1</sup> Percentage of internalization (macrophages) or invasion (fibroblasts) considered as % recovered CFU of the inoculum as described in Materials and Methods. Data represent the mean ± standard error of the mean from three independent experiments.

<sup>2</sup> Mean ± standard error of three independent experiments is shown.

## Figure legends

**Fig 1. Growth curves of the parent and mutant strains in LB.** Both strains displayed identical growth responses (OD<sub>600</sub>, LB broth culture). Empty circles represent WT; solid squares mutant strains.

**Fig 2. Infection of SIM and SIF with *S. Choleraesuis*.** (A) Survival of WT(●),  $\Delta phoP$ (□),  $\Delta rpoS$ (○) and SC-54(■) inside SIM. Data are expressed as the mean of three independent replicated experiments  $\pm$  standard error of the mean. (B) Survival of WT(●),  $\Delta phoP$ (□),  $\Delta rpoS$ (○) and SC-54(■) inside SIF. Mean  $\pm$  standard error of the mean (SEM) for three independent replicated experiments are shown. Asterisks and crosses indicate statistically significant differences. \* $p$  when compared with SC-54 and † $p$  when compared with WT (\*, †,  $p < 0.05$ ; \*\*, ††  $p < 0.001$ ).

**Fig. 3. Immunofluorescence microscopy of SIF infected with *S. Choleraesuis*.** (A) Representative images of WT,  $\Delta phoP$  and  $\Delta rpos$  inside fibroblasts 24 h post-infection. (B) Massive intracellular proliferation of WT inside a dead fibroblast. *S. Choleraesuis* (green), nuclei (blue), dead cell (red).

**Fig. 4. Number of intracellular *Salmonella* inside SIF at 2 and 24 hours post-infection.** SIF were infected with the WT (black bar),  $\Delta phoP$  (grey bar) and  $\Delta rpoS$  (white bar). Bacteria were immunostained with rabbit *Salmonella* O antiserum and the number of intracellular bacteria was determined by counting using immunofluorescence microscopy. The graphs show the percentage of live cells infected with different numbers of bacteria (1–5, 6–20 and > 20) resulting from three independent experiments. A total of 100 live



infected cells from each experiment was counted. Statistical analyses were performed using Student's *t*-test; \* $p < 0.05$  considered significant; \*\* $p < 0.01$ , very significant; \*\*\* $p < 0.001$ , extremely significant.

Accepted Manuscript

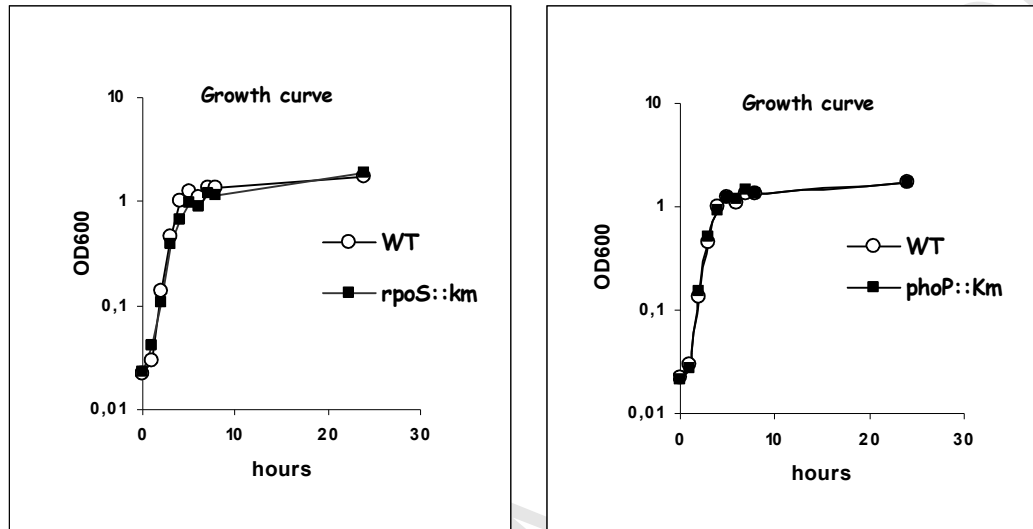
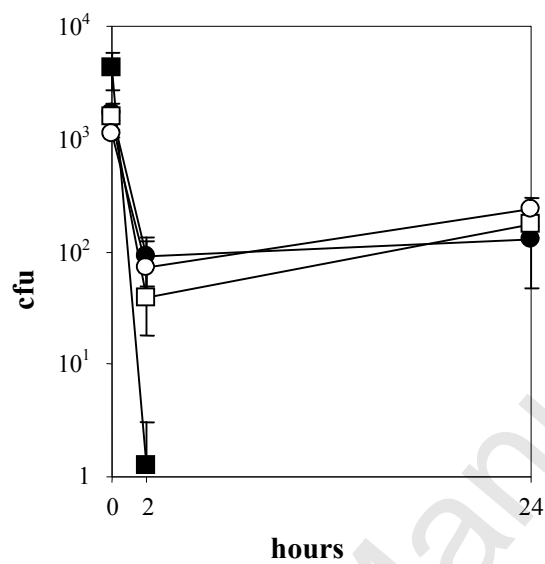


Figure 1

A



B

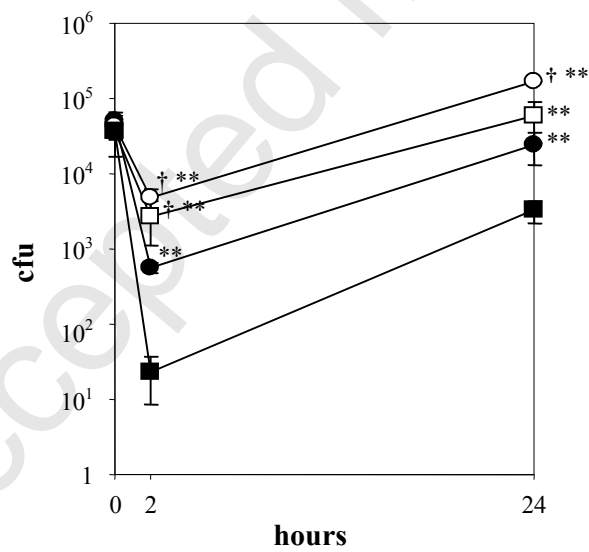
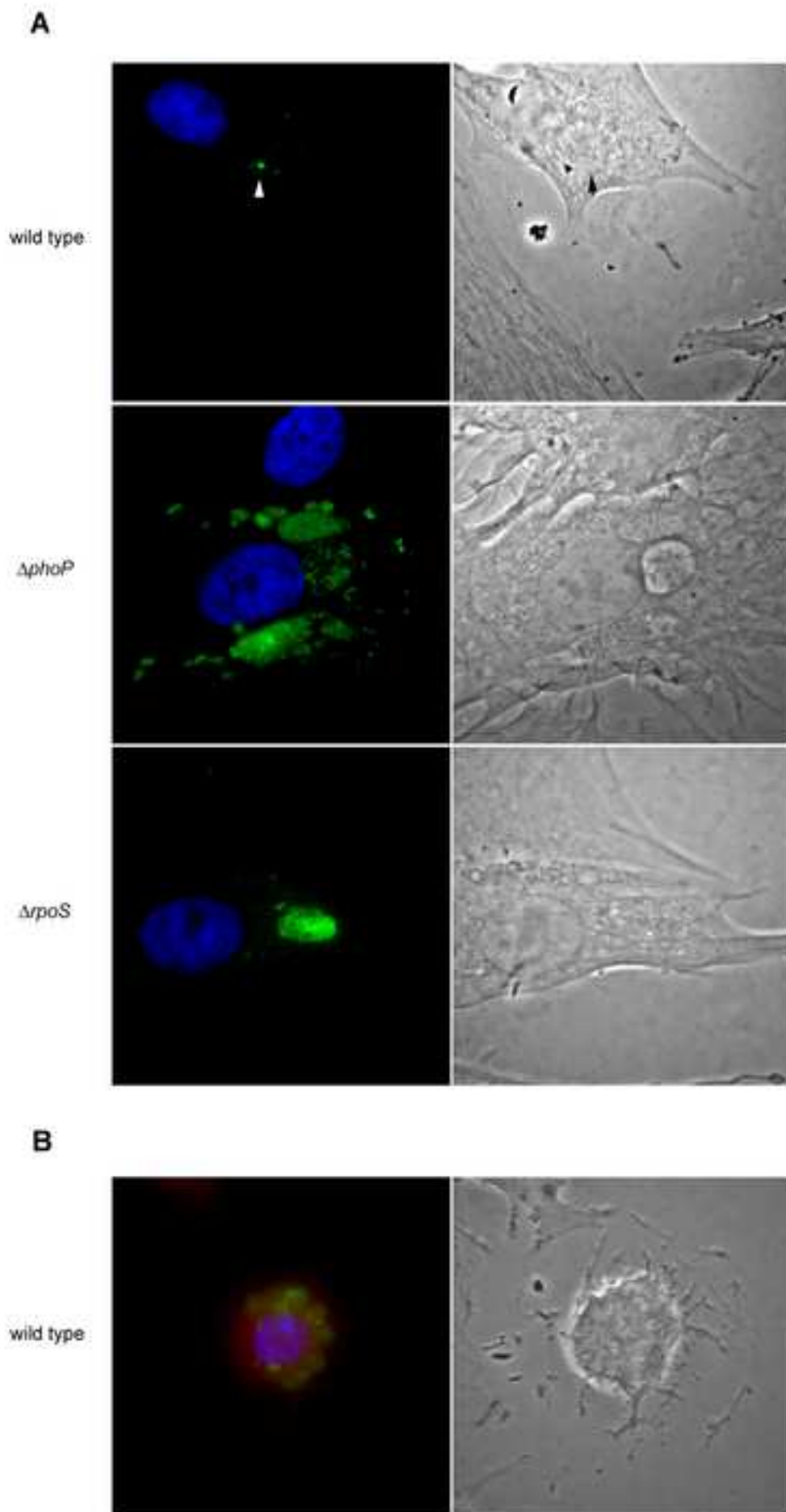
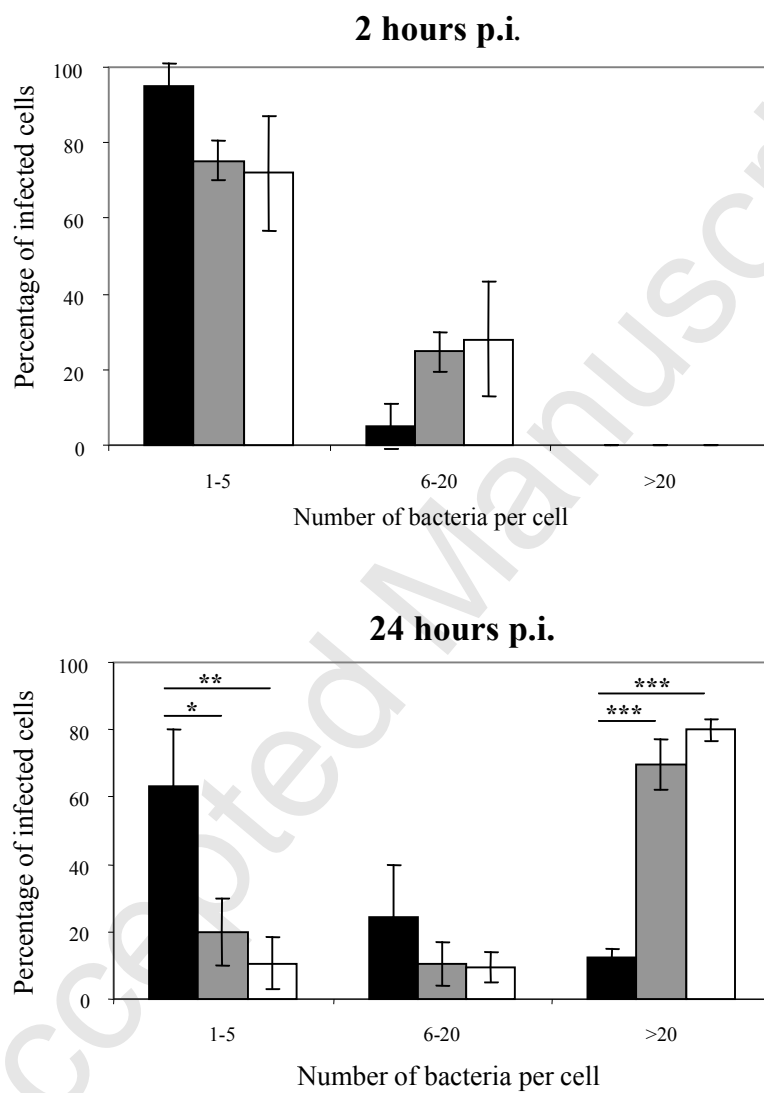


Figure 2



**Figure 4**